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Downregulated gene expression of TGF-βs in diabetic oral wound healing

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ABSTRACT

Background: Healing of tooth extraction sockets in poorly controlled diabetic patients is often delayed and accompanied by severe infection. The exact cellular and molecular mechanisms underlying the pathogenesis of this complication are still not fully understood.
Objectives: The purpose of this study was to investigate molecular changes associated with delayed oral wound healing in diabetes.
Materials and methods: Six to eight weeks old male type 2 diabetes and age matched control inbred mice were used and maxillary molar tooth extractions were performed. At 4 and 7 days after tooth extraction, the edentulous mucosa of the mice were harvested, and analyzed for histology and gene expression of key wound healing factors.
Results: In the diabetic model, histological analysis showed that epithelial tissue migration for wound closure was delayed after tooth extraction compared to the control. Quantitative real-time PCR revealed that expression of the TGF-β1, TGF-β2, TGF-β3, TGFβRII and TGFβRIII genes was significantly down-regulated in the diabetic model at 4 and 7 days after tooth extraction.
Conclusion: These results suggest that delayed wound healing of oral mucosa in diabetes may be associated with decreased expression levels of these regulatory genes which play important roles in controlling epithelial wound closure.

1. Introduction

Diabetes mellitus affects more than 21 million people in the United States (American Diabetes Association website) and 180 million people worldwide (World Health Organization website). Most patients have non-insulin dependent (type 2) rather than insulin dependent (type 1) diabetes. Several clinical and animal studies have shown a positive correlation between type 2 diabetes and periodontal disease (He et al., 2004); impaired extra-oral/skin wound healing (Peppa et al., 2003); and the development of peri-radicular lesions (Iwama et al., 2003).

Delayed healing of cutaneous wounds in diabetic patients is associated with elevated inflammatory responses, characterized by specific cytokine expression and recruitment of immunocompetent inflammatory cells (Wetzler et al., 2000; Slavkovsky et al., 2011). Furthermore, reports have shown diabetic wounds have alterations in the finely balanced expression of various growth factors, including transforming growth factor β (TGF-β) (Bitar and Labbad, 1996), matrix metalloproteinases (MMPs) (Lobmann et al., 2002), insulin-like growth factor 1 (Brown et al., 1997), platelet-derived growth factor (Castronuovo et al., 1998), nerve growth factor (Fernyhough et al., 1995), keratinocyte growth factor (Werner et al., 1994), interleukin 6 (IL-6) (Fahey et al., 1991; Ebaid et al., 2011), and vascular endothelial growth factor (VEGF) (Altavilla et al., 2001).

Intra-orally, healing of tooth extraction sockets in poorly controlled diabetic patients is often delayed and accompanied by severe infection (Devlin et al., 1996; Nakamura et al., 2002). Diabetic patients may feature a reduced level of salivary epithelial growth factor which may contribute to the development of oral and systemic complications of diabetes (Oxford et al., 2000). The negative effect on early osseointegration of dental implants was shown in diabetic pigs (Schlegel et al., 2011). The exact cellular and molecular mechanisms underlying the pathogenesis of this complication are still not fully understood.

We investigated the regulation of cytokines in diabetic oral wounds which might be related to decreased angiogenesis, diminished recruitment of smooth muscle cells, and delayed wound healing, using a type 2 diabetic mouse model. We

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hypothesized that local cytokine expression might be related with delayed oral wound healing in diabetes. Quantitative real-time polymerase chain reaction (PCR) was used to measure gene expression for proinflammatory and angiogenic cytokines that play important roles in mucosal wound healing.

2. Materials and methods

2.1. Animal models

Six to eight weeks old male type 2 diabetes BKS.Cg-m +/+ lepr^{db} (db/db+/+); $n = 10$ and age matched control C57BLKS/J inbred mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA). The db/db (+/+) mice carry a single autosomal recessive mutation on chromosome 4 and exhibit characteristics similar to those of human type 2 diabetes. This mouse strain is a common animal model for type 2 diabetes and has been used for many studies of impaired wound healing (Brem et al., 2007; Michaels et al., 2007). All animal procedures have been approved by Harvard Medical School office of Animal Protection.

2.2. Measurement of glucose levels and body weight

Baseline body weight was measured and glucose levels were obtained from both diabetic and control mice using glucometer (LifeScan, Inc., Milpitas, CA, USA).

2.3. Teeth extraction

Animals were anesthetized by intramuscular injection with ketamine (75 mg/kg body weight) and xylazine (5–10 mg/kg body weight). Extractions of maxillary right molars in both groups were performed with a dental explorer following the previously published protocol (Nishimura et al., 1987) (Fig. 1). The maxillary left molar non-extraction site was served as a control. The mice were sacrificed at 4 and 7 days after tooth extraction.

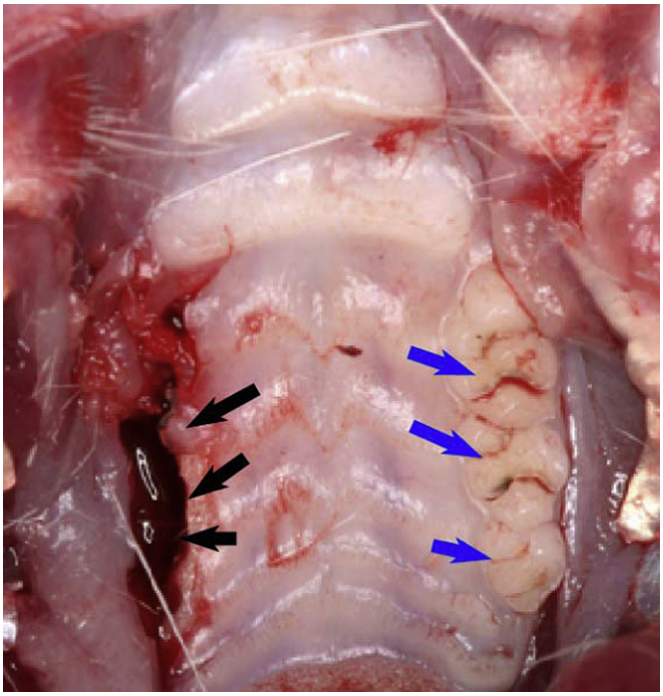

Fig. 1. Extraction of maxillary molars (black arrows); the control site (non-extracted site, blue arrows).

2.4. Histological analysis

Following sacrifice, the head of each animal was dissected free and then placed for 48 h in cold 4% paraformaldehyde in phosphate-buffered saline followed by dissection of the palate with intact edentulous mucosa (EOM) and the untreated gingiva (Sukotjo et al., 2002; Reichenberger et al., 2000). The tissues were decalcified for 7 days in Cal-Ex (Fischer Scientific, Fair Lawn, NJ, USA) and embedded in paraffin. The mounted 5 μm sections were stained with hematoxylin and eosin (H&E). The parameters evaluated from H&E staining were the degree of re-epithelialization, density of granulation tissue, and inflammatory cell infiltration.

2.5. Quantitative real-time PCR

Both the tissue, EOM and untreated gingiva, from diabetes and control mice were homogenized separately. Total RNAs were isolated from these tissues using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). Mouse Universal ProbeLibrary probes and target-specific PCR primers for type I collagen (Col-1), IL-1β, MMP-8, MMP-9, VEGF-B, TGF-β1, TGF-β2, TGF-β3, TGF-β receptor I (TGFβRI), TGFβRII, TGFβRIII, β-glycan, and β-actin were selected using the ProbeFinder assay design software (Table 1). All assays were prepared using standard conditions in a master mix solution without any effort for assay optimization. For each data point, there were three replicates. cDNA was synthesized from 1 μg of total RNA for each sample using reverse transcriptase (Roche, Nutley, NJ, USA). The primers were used to confirm relative changes in mRNA levels by quantitative real-time PCR, using the

Table 1
Primer sequences for QRT-PCR.

Col-1	Forward	TGGAGATGATGGGGAAGC
	Reverse	AATCCACGAGCACCCTGA
IL-1β	Forward	TGTAATGAAAGACGGCACACC
	Reverse	TCTTCTTTGGGTATTGCTTGG
MMP-8	Forward	AATGGCATTCAGACAATCTATGG
	Reverse	CTCAGGTGGGGGTCACAG
MMP-9	Forward	ACGACATAGACGGCATCCA
	Reverse	GCTGTGGTTCAGTTGTGGTG
VEGF-B	Forward	AGTCAGCTGGGGGAGATGT
	Reverse	GGGGTATGGCAACCCTGT
TGF-β1	Forward	TGGAGCAACATGTGGAACTC
	Reverse	GTCAGCAGCCGGTTACCA
TGF-β2	Forward	TGGAGTTCAGACACTCAACACA
	Reverse	AAGCTTCGGGATTTATGGTGT
TGF-β3	Forward	CCCTGGACACCAATTACTGC
	Reverse	TCAATATAAAGGGGGCGTACA
TGFβRI	Forward	AATGTTACGCCATGAAAATATCC
	Reverse	CGTCCATGTCCCATTGTCTT
TGFβRII	Forward	GGCTCTGGTACTCTGGGAAA
	Reverse	AATGGGGGCTCGTAATCCT
TGFβRIII	Forward	TCCAAACATGAAGGAGTCCA
	Reverse	GTCCAGGCCGTGGAAAAT
β-Glycan	Forward	TCCAAACATGAAGGAGTCCA
	Reverse	GTCCAGGCCGTGGAAAAT
β-Actin	Forward	GTGGTACGACCAGAGGCATAC
	Reverse	AAGGCCAACCGTGAAAAGAT

480 LightCycler (Roche). Reactions were performed in 20 μl reaction volumes for the genes encoding Col-1, IL-1β, MMP-8, MMP-9, VEGF-B, β-glycan, TGF-β1, TGF-β2, TGF-β3, TGFβRI, TGFβRII, TGFβRIII, and β-actin using 1 μl of cDNA under the following conditions: 95 °C for 5 min, 50 cycles for 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 s.

The method used for obtaining quantitative data of relative gene expression was the comparative Ct method (also as known the $2^{-\Delta\Delta Ct}$ method) (Schmittgen and Livak, 2008). Statistical differences among the groups were assessed by one-way analysis of variance (ANOVA) and the analyses were repeated over different genes. A significance level of 0.05 was used in all statistical comparisons. Post-hoc analysis using Scheffe method was performed to detect pairs of groups with statistical difference. All statistical analyses were performed using PASW Statistics 18.0 (SPSS Inc., Chicago, IL, USA).

3. Results

3.1. Glucose levels and body weight

Baseline body weight of diabetic mice was 2 times larger than the control group (diabetic: 38.8 ± 3.3 g, control: 17.7 ± 1.3 g). Glucose levels were obtained (diabetes: 300 ± 27 mg/dl, control: 115 ± 7 mg/dl) and found to be significantly higher for the diabetic mice.

3.2. Histological analysis of oral wound healing process in a diabetic model

The results of histological analysis showed that epithelial tissue migration in diabetic mouse samples at 4 days after tooth extractions was delayed. These samples showed a larger epithelial gap at the extraction site (Fig. 2B) and delayed inflammatory response (Fig. 2D), compared to control (Fig. 2A & C). An elevated inflammatory response, characterized by recruitment of immunocompetent inflammatory cells (lymphocytes) was seen in the epithelium and connective tissues of diabetic samples (Fig. 2F) at 7 days compared to control (Fig. 2E).

3.3. Gene expression levels in diabetic oral wounds

Quantitative real-time PCR was used to quantify the mRNA levels of several of the major known mediators that are related with wound healing. Fig. 3 shows gene expression levels in diabetic and control oral wounds. The gene expression levels of Col-1 (Fig. 3A), MMP-9 (Fig. 3D), and VEGF (Fig. 3E) in both the control and diabetic group similarly increased at 4 and 7 days after extraction compared to 0 day. At 0 day, the IL-1β (Fig. 3B) and mRNA level in the diabetic group were significantly lower than the control. Conversely, at 7 days, IL-1β (Fig. 3B) and MMP-8 (Fig. 3C) gene expressions in the diabetic group were significantly higher than the control. The β-glycan level in the diabetic group was significantly higher at 0 day and lower at 7 days than the control (Fig. 3F). The TGF-β1 gene expression in the diabetic group was significantly more downregulated compared to the control at all time points (Fig. 3G). Also, the gene expressions of TGF-β2 (Fig. 3H), TGF-β3 (Fig. 3I), TGFβRII (Fig. 3K), and TGFβRIII (Fig. 3L) in the diabetic group were significantly downregulated compared to the control at 4 and 7 days. There was no significant difference in TGFβRI mRNA level between the diabetic and the control groups at any of the time points (Fig. 3J). All quantitative real-time PCR results are summarized in Table 2.

4. Discussion

In this study, we used a type 2 diabetes BKS.Cg-m +/+ leprdb (db/db+/+) animal model. The db/db (+/+) mice carry a single autosomal recessive mutation on chromosome 4 and exhibit characteristics similar to those of human type 2 diabetes. This mouse strain is a common animal model for type 2 diabetes (Wang et al., 2011) and impaired wound healing studies (Brem et al., 2007; Michaels et al., 2007).

Generally, normal wound healing in the oral mucosa is clinically distinguished from dermal healing in terms of both its rapid healing and lack of scar formation. Although there has been considerable study of the skin wound healing of genetically diabetic mice (Brown et al., 1997; Sullivan et al., 2004; Chen et al., 2005; Sharma et al., 2006; Andrikopoulou et al., 2011), there has been no report about oral wound healing in this model. The results of the present study are the first report of diabetic oral wound healing using a tooth extraction model. We demonstrated that wound healing of oral epithelium was delayed after the tooth extractions in a diabetic model. In healthy wound-healing following tooth extraction, the initial restoration of epithelial integrity is complete within 4–7 days (Kapur and Skhlar, 1963). Adaptation of the oral mucosal tissue within the tooth extraction area often results in the generation of distinct epithelial and connective tissue (Wirthlin et al., 1984). In our diabetic extraction model, epithelial tissue migration was delayed, and showed a larger epithelial gap at the extraction site at 4 days and less inflammatory response compared to the control. At 7 days, an elevated inflammatory response, characterized by recruitment of lymphocytes, was still seen in the epithelium and connective tissues of diabetic samples as compared to the control. These results indicated that recruitment of inflammatory cells was delay and subsequently inflammation remained in diabetic oral wounds.

Diabetic skin wounds demonstrate impairments not only in the initial recruitment of inflammatory cells following injury (Goova et al., 2001) but also in the bactericidal activity of neutrophils and other leukocytes (Rayfield et al., 1982). Once infiltration is finally established, wounds retain a high concentration of neutrophils and macrophages, leading to sustained increases in the proinflammatory cytokines IL-1β and tumor necrosis factor α (Wetzler et al., 2000). Like diabetic skin wounds, impairments were observed in the initial recruitment of inflammatory cells following tooth extraction in our oral diabetic model and the wounds retained a high concentration of mononuclear cells. These events in the diabetic oral wounds might be associated with increased IL-1β gene expression at 7 days. Conversely, it has been reported that there were fewer inflammatory cells in normal oral wounds correlating with the decreased expression of IL-6, which was responsible for the reduced recruitment of neutrophils and macrophages (Szpaderska et al., 2003).

Three isoforms of TGF-β (TGF-β1, -2, and -3) are found in mammals and their timing of appearance, duration and sites of expression, together with those of their receptors, show a distinct pattern during normal acute wound healing (Levine et al., 1993). Several studies have examined the role of TGF-β1 in impaired healing and diabetic skin (Frank et al., 1996; Jude et al., 2002; Al-Mulla et al., 2011). TGF-β1 has wide ranging functions in the normal dermal healing process (O'Kane and Ferguson, 1997). In humans with venous ulcers, chronic pressure wounds and diabetic foot ulcers, the normal elevation of TGF-β1 found in acute wound healing was absent (Jude et al., 2002; Schmid et al., 1993). In diabetic foot ulcers, TGF-β1 protein levels were found to be reduced, whereas TGF-β2 and TGF-β3 were elevated (Jude et al., 2002). Even in unwounded diabetic skin, TGF-β1 was lower than in normal skin. In a normal oral wound study, decreased expression of TGF-β1 was seen compared with normal skin wounds (Szpaderska et al., 2003).

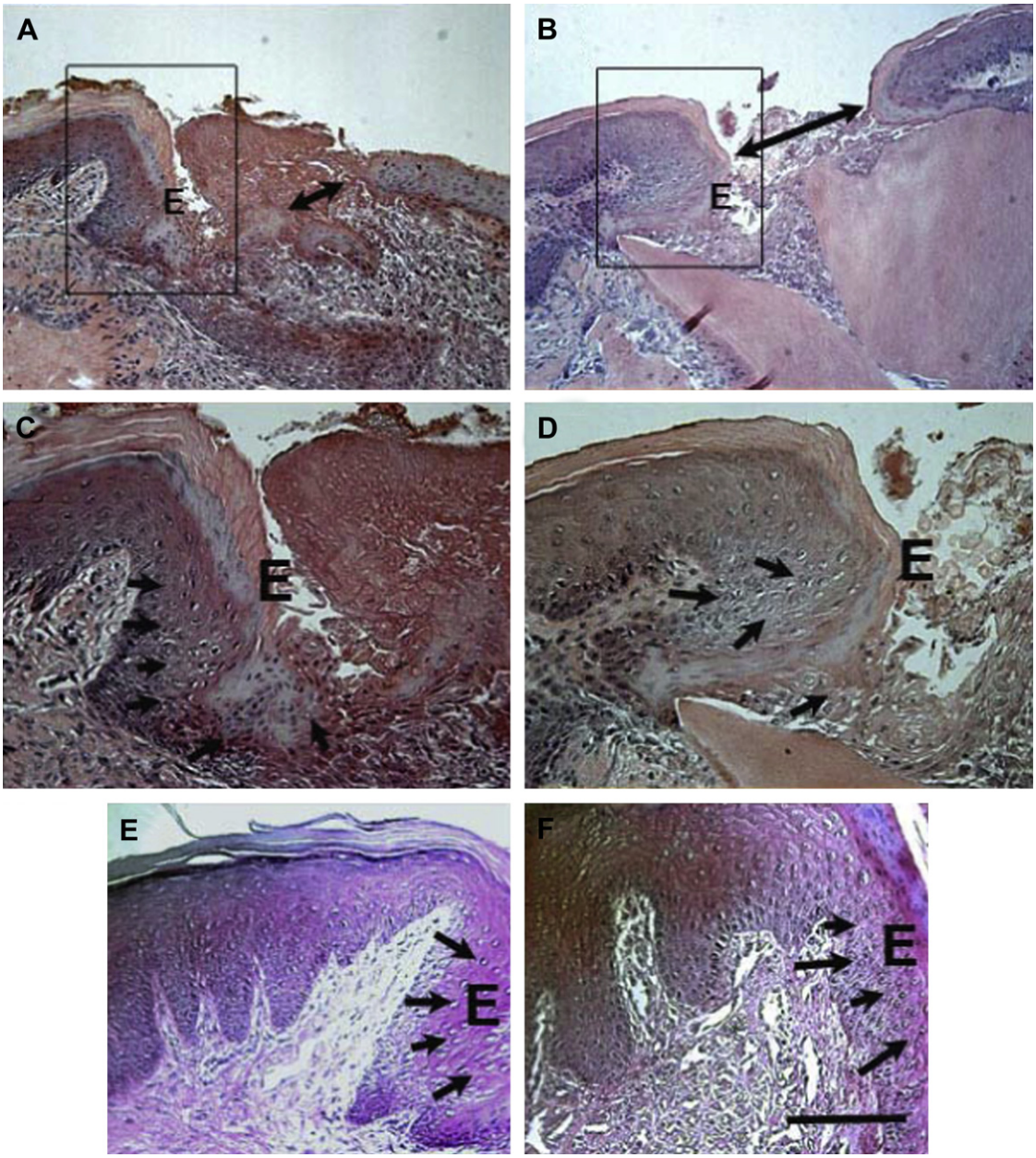

Fig. 2. Histological appearance (H&E) of healing of the extraction site at 4 (A–D) and 7 days (E & F) in control and diabetic mouse, respectively. Control, (A) ×20, (C) ×40 and (E) ×40; Diabetes, (B) ×20, (D) ×40, and (F) ×40. E: Extraction site. Double allows shows epithelial gaps. Single arrows indicate inflammatory cells. Bar = 100 μm.

Analysis of our data in the normal oral wounds showed that there was no change in TGF-β1 mRNA levels during healing. This data suggests that TGF-β1 may not be necessary to mediate normal oral wound healing. In contrast, TGF-β1 mRNA levels in the diabetic oral wounds decreased at 4 days and remained downregulated until 7 days. These levels, including basal data in diabetes, were significantly lower at all time points compare to those in the control oral mucosa. Also, TGF-β2 expression in diabetes was significantly decreased at 4 days compared with control. These results suggest that the decreased levels of TGF-β1 and TGF-β2 mRNA expression in the diabetic oral wounds may be sufficient to allow for wound closure. In our findings, TGF-β3 mRNA levels in the control oral wounds increased slightly at 4 days and then significantly upregulated maximally at 7 days. In contrast, TGF-β3 mRNA levels in the diabetic oral wounds had no change and were significantly lower at 7 days compared to those in the control. This finding suggests that the decreased levels of TGF-β3 mRNA expression in the diabetic oral wounds may be associated with delayed epithelial closure. In another study, the ratio of TGF-β3 to TGF-β1 was high in healing wounds with improved scarring (O'Kane and Ferguson, 1997). TGF-βs are also known as an intraocullary synthesized antiangiogenic factor (Simó et al., 2006). In a diabetes model, gene expression levels of VEGF, which plays an important role in angiogenesis (Seitz et al., 2011), were similar to the control. Therefore, impaired oral wound healing in diabetes may not be associated with neovascularization/neoangiogenesis.

The TGF-β receptors (TGFβRI, -II, and -III), may also play an important role in wound healing. All three TGF-β isoforms bind to TGF-β receptors, but with varying affinity, possibly resulting in specific responses or functions (Derynck and Feng, 1997). During acute wound healing in normal mice, mRNA levels for both TGFβRI and TGFβRII in skin wounds were elevated (Frank et al., 1996).

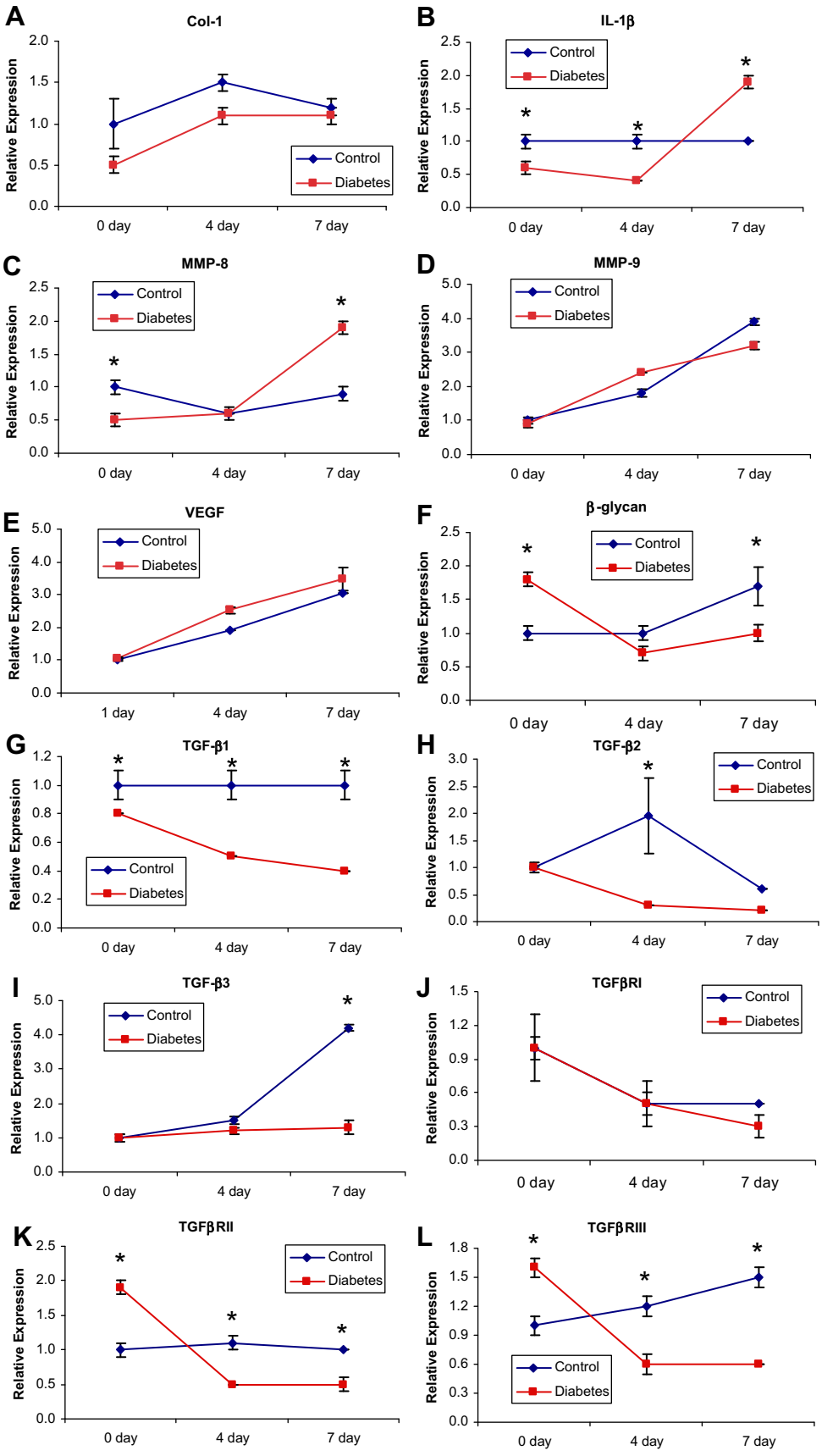

Fig. 3. mRNA levels of edentulous oral mucosa and untreated gingival from diabetic and control mice. cDNA was synthesized from 1 μg of total RNA for each sample using reverse transcriptase (Roche, Nutley, NJ, USA). The primers were used to confirm relative changes in mRNA levels by quantitative real-time PCR, using the 480 LightCycler (Roche). Reactions were performed in 20 μl reaction volumes using 1 μl of cDNA under the following conditions: 95 °C for 5 min, 50 cycles for 95 °C for 10 s, 60 °C for 15 s, and 72 °C for 1 s. Quantitative real-time PCR for experimental genes were normalized against internal control (β-actin). Triplicates of each data point were averaged, and the mean values were used for statistical analysis. Data are expressed relative to the level of the untreated gingiva from control mice at day 0, which was set at 1. mRNA expression levels of type I collagen (Col-1) (A), interleukin 1β (IL-1β) (B), matrix metalloproteinase 8 (MMP)-8 (C), MMP-9 (D), vascular endothelial growth factor B (VEGF) (E), β-glycan (F), transforming growth factor β 1 (TGF-β1) (G), TGF-β2 (H), TGF-β3 (I), TGF-β receptor 1 (TGFβRI) (J), TGFβRII (K), and TGFβRIII (L). Results are expressed as mean ± SD. *Significant difference between normal and diabetic groups ($p < 0.05$).

Table 2
Summary of quantitative real-time PCR results.

		0 Day	4 Day	7 Day
Col-1	Control	1.0 ± 0.3	1.5 ± 0.1	1.2 ± 0.1
	Diabetes	0.5 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
IL-1β	Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.0
	Diabetes	*0.6 ± 0.1	*0.4 ± 0.0	*1.9 ± 0.1
MMP-8	Control	1.0 ± 0.1	0.6 ± 0.1	0.9 ± 0.1
	Diabetes	*0.5 ± 0.1	0.6 ± 0.0	*1.9 ± 0.1
MMP-9	Control	1.0 ± 0.1	1.8 ± 0.1	3.9 ± 0.1
	Diabetes	0.9 ± 0.1	2.4 ± 0.0	3.2 ± 0.1
VEGF	Control	1.0 ± 0.0	0.3 ± 0.1	0.5 ± 0.0
	Diabetes	0.2 ± 0.0	0.5 ± 0.1	0.6 ± 0.1
β-Glycan	Control	1.0 ± 0.1	1.0 ± 0.1	1.7 ± 0.3
	Diabetes	*1.8 ± 0.1	0.7 ± 0.1	*1.0 ± 0.1
TGF-β1	Control	1.0 ± 0.1	1.0 ± 0.1	1.0 ± 0.1
	Diabetes	*0.8 ± 0.0	*0.5 ± 0.0	*0.4 ± 0.0
TGF-β2	Control	1.0 ± 0.0	2.0 ± 0.7	0.6 ± 0.0
	Diabetes	1.0 ± 0.1	*0.3 ± 0.0	0.2 ± 0.0
TGF-β3	Control	1.0 ± 0.1	1.5 ± 0.1	4.2 ± 0.1
	Diabetes	1.0 ± 0.1	1.2 ± 0.1	*1.3 ± 0.2
TGFβRI	Control	1.0 ± 0.3	0.5 ± 0.2	0.5 ± 0.0
	Diabetes	1.0 ± 0.1	0.5 ± 0.1	0.0 ± 0.1
TGFβRII	Control	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1
	Diabetes	*1.9 ± 0.1	*0.5 ± 0.0	*0.5 ± 0.1
TGFβRIII	Control	1.0 ± 0.1	1.2 ± 0.1	1.5 ± 0.1
	Diabetes	*1.6 ± 0.1	*0.6 ± 0.1	*0.6 ± 0.0

*Significant difference between normal control and diabetic groups ($p < 0.05$).

However, a lack of TGFβRI elevation was discovered in chronic diabetic ulcers (Jude et al., 2002). The results of our study showed that gene expression of TGFβRII and TGFβRIII in the oral diabetic wounds decreased significantly compared to the control wounds. In contrast, there was little difference in TGFβRI expression patterns in wound beds between diabetic and normal models. Thus, the deficiency of TGF-β1 and TGF-β3 seen in diabetic oral wounds may result in considerable disruption to the normal healing process because of both their direct roles, and amplification by reduction of TGFβRII and TGFβRIII expression.

Like skin wound healing, our studies demonstrated that diabetic oral wound healing was delayed compared with normal oral wound healing. Our results suggest that delayed wound healing of oral mucosa in diabetes may be associated with decreased levels of those cytokines and their receptors which play important roles in epithelial wound closure, especially TGF-β1, TGF-β2, TGF-β3, TGFβRII and TGFβRIII. Although there have been few reports describing reduced levels of TGF-βs and their receptors in diabetic oral wounds, the results of this study may provide information to help develop future clinical therapies for diabetic patients. A recent study suggested that the administration of TGF-β3 induced rapid re-epithelialization, suppressed scar formation, favorable restoration of hyaluronic acid and elastin (Ohno et al., 2011). Therefore, these downregulated genes may be new targets for molecular therapeutics to encourage wound healing of oral mucosa in diabetes patients in the future.

5. Conclusion

Histologically, epithelial tissue migration for wound closure was delayed after tooth extraction in the diabetic model compared to the control. Gene expression of the TGF-βs and TGF-β receptors was significantly downregulated in the diabetic model at early time points after tooth extraction. These results suggest that delayed wound healing of oral mucosa in diabetes may be associated with decreased expression levels of these regulatory genes which play important roles in controlling epithelial wound closure.

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